COMPLEX IMMUNO-GENE MEDICAL COMPOSITION FOR INHIBITING TUMOR CELLS

BACKGROUND OF THE INVENTION

1.Field of the Invention

[0001] The present invention relates to a medical composition for inhibiting tumor cell growth. In particular, it relates to a complex ~~immuo~~immuno-gene medical composition for inhibiting tumor cell growth~~,~~ and a method for inhibiting tumor cell growth using ~~the~~ such medical composition ~~thereof~~.

2. The Prior Arts

[0002] Canine transmissible venereal tumor (CTVT) is a naturally ~~occurred~~ occurring, poorly ~~differential~~ differentiated tumor cell~~s~~. The growth of the CTVT is similar to an allograft. CTVT ~~in Canines~~ are transmitted ~~to each other~~ in Canines~~,~~ by mating, biting or contacting, ~~by~~ wherein viable tumor cells enter through injured skin or mucus.

[0003] In an experimental model, CTVT shows an ~~expectable~~ expected growth pattern. The growth phases in the CTVT model include a Progressive phase (P phase), a Stasis phase (S phase), and a Regressive phase (R phase). CTVT expresses ~~little~~ few major histocompatibility (MHC) molecules in the P phase but a large amount of transforming growth factor-β (TGF-β) in the P phase and the R phase. TGF-β is capable of inhibiting expression of MHC I and MHC II, and inhibiting activity of natural killer (NK) cells. Besides, 85 % of tumor infiltrating lymphocytes (TILs) ~~of~~ in CTVT are lymphocytes ~~being~~ that are non-T or non-B cells, which are not able to express antigens characterized as T-cells and B-cells. From morphological observation, the aforementioned cells contain ~~big~~ large granules in their cytoplasm similar to those found in NK cells. It is presumed that the non-T or non-B cells ~~shall be~~ are NK cells.

[0004] Major histocompatibility complex (MHC) class I antigens are 44 kDa glycoproteins expressed on the cell plasma membrane associated with β_2-

microglobulin (β_2m), and ~~it also called~~ they are also known as histocompatibility leukocyte antigens (HLA) in humans.

[0005] Tumor cells grow by escaping the monitoring of the host immune system through many different mechanisms. One of the mechanisms used by tumor cells is ~~no~~ a lack of or a low expression of MHC class I antigens. For examples, Human tumor cells including primary breast carcinoma, advanced renal cell carcinoma, melanoma, prostate cancer, lung carcinoma, and other tumor sources from colon, bladder, skin~~,~~ and endometrium have been found to express low MHC levels, and some cancer cells even express no MHC. In animals, low expression of MHC is also found in T lymphoma caused by Marek's disease virus in ~~poultries~~ poultry and CTVT ~~occurred~~ occuring in canines.

[0006] According to the "missing self" hypothesis, when tumor cells express low or no MHC, the activating receptors on the surface of the natural killer (NK) cells in the host are activated, and the NK cells recognize and kill the target cells. However, many tumor cells secrete transforming growth factors to inhibit the cytotoxicity of NK cells. Therefore, the ~~incapability~~ inability of host immune system to function normally is one of the reasons for the tumor cells to grow rapidly without much ~~of the~~ constraint.

[0007] TGF-β is a 25 kDa homodimerric protein with very potent pleiotropic regulatory effects on the mammalian immune system. Addition of exogenous TGF-β to a culture of lymphocytes decreases the proliferation of B cells, mature T cells, thymocytes, NK cells and lymphokine-activated killer (LAK) cells. Currently, It is known that many tumor cells including colorectal cancer, mammary tumor, thyreoglandular cancer, hepatocellular carcinoma (HCC) and Meth A tumor produce TGF-β . TGF-β is capable of ~~helping~~ aiding in the growth of tumors by enhancing angiogenesis and cell adhesion. Moreover, the tumor cells evade the host immune surveillance by low MHC and low intercellular adhesion molecule-1(ICAM-1) expressions.

[0008] An antagonism or reduced secretion of TGF-β is a possible way to restore the normal function of a host immune system and fight against tumor cells. Methods of immuno-therapy ~~to inhibit~~ for inhibiting TGF-β are presented herein~~.~~

~~a~~Administration of TGF-β antibody by injection and gene therapy with antisense oligonucleotide are the other two major methods.

[0009] T lymphocytes cannot function to kill tumor cells that ~~secret~~ secrete TGF-β or express no or low MHC. Accordingly, NK cells play important roles ~~in~~ against tumor cells expressing no or low MHC. NK cells differentiation-related cytokines ~~are~~ have proved to be successful in ~~removal of~~ removing tumor cells. Those cytokines including γ- interferon (INF-γ), interleukin-2 (IL-2), IL-12, IL-15, IL-18 and IL-21. They are related to the functions of T lymphocytes, B lymphocytes, NK cells and other immune cells.

[0010] It is demonstrated that several cytokines are effective ~~in~~ against tumor cells in some in vitro and animal experiments. Those researches ~~draw great attention to apply~~ focus upon the application of cytokines to cancer therapy. There are two kinds of cytokines that have been used against cancer cells. One kind is T-helper type 1 (Th1) cytokine, which stimulates reactions related to IL-2 and INF-γ production, and to the following cellular immunity, including IL-2, IL-12, IL-15, IL-18 and INF-γ. Another kind is T-helper type 2 (Th2) cytokine, which stimulates reactions related to production of IL-4, IL-5 and IL-6 (these three cytokines stimulate B lymphocytes to grow and differentiate), and which induces humoral immunity. However, results from those clinical researches using immuno-therapy of cytokines reveal that factors such as the ~~way~~ method of administration, ~~administrated~~ the amount administered, kinds of tumors, other additional cytokines or drugs, or potential side effects, and so on, are important in therapeutic effectivity and clinical application. Although immuno-gene-therapy is effective against tumor cells, there ~~is~~ are limitations in its practical application for ~~the~~ conventional methods. Usage of a combination of various cytokines according to the immune characteristics of tumor cells may be a more applicable method ~~to~~ in the fight against tumors.

SUMMARY OF THE INVENTION

[0011] A primary object of the present invention is to provide a complex immuno-gene medical composition for inhibiting the growth of tumor cells. The composition is capable of restoring the ~~cytotocicity~~ cytotoxicity of NK cells by antagonizing the

TGF-β inhibitory effect on the host immune system.

[0012] Another object of the present invention is to provide a complex immuno-gene medical composition~~,~~ which further activates the ~~cytotocicity~~ cytotoxicity of NK cells in a host immune system.

[0013] Some tumor cells express low MHC to escape ~~the~~ specific attack from the host immune system (CD8+ T lymphocytes) in the growth period, but the low or no expression of MCH activates NK cells. In the meanwhile, tumor cells secrete a high level of TGF-β to inhibit differentiation and activity of NK cells. Moreover, TGF-β reduces the numbers and inhibits the cytotoxicity of NK cells, lowers the expression of IFN-α and the α chain of the IL-2 receptor on the cellular surface, and reduces the secretion of INF-γ.

[0014] The present invention provides a complex ~~immune~~immuno-gene medical composition according to the aforementioned mechanism used by tumor cells to evade the host immune surveillance. The composition is capable of activating the immune system by activating NK cells. The composition ~~is the usage of~~ uses a combination of ~~a plurality of~~ cytokines, that is, the ~~combined usage~~ combination of ~~the kinds of~~ Th1 and Th2 cytokines. Th2 cytokines antagonize TGF-β inhibiting NK cells to disable the inhibition of the immune system, and Th1 cytokines activate NK cells in the host to enhance ~~the~~ their ability to ~~fighting~~ fight against tumor cells. By means of the complex immuno-gene medical composition, removal of tumor cells is ~~expectable~~ expected.

[0015] The ~~aforementioned~~ Th1 cytokines, as used herein, include IL-2, IL-12, IL15, IL-18 and INF-γ, and so on. ~~And~~ tThe ~~aforementioned~~ Th2 cytokines, as used herein, include IL-4, IL-5 and IL-6, and so on.

[0016] To demonstrate the inhibition effect of the composition according to the present invention ~~to~~ on tumor cells, CTVT is used as a tested tumor model. Some reasons for choosing CTVT as a tested tumor model are ~~described in the followings~~ as follows: (1) CTVT is a ~~kind~~ type of tumor expressing low MHC; (2) CTVT produces a lot of TGF-β molecules; and (3) about 85% of tumor infiltrating lymphocytes (TIL)

isolated from CTVT, expressing no CD3 and CD21, and ~~is~~ which are not T cells or B cells, are presumed ~~as~~ to be NK cells of canines.

[0017] Firstly, a plasmid containing human IL-6 coding sequence and another plasmid containing human IL-15 coding sequence are constructed with conventional methods, respectively. The ~~S~~sequences of the constructed plasmids are then confirmed. Expressed IL-6 and IL-15 is recovered after transfection *in vitro* and tested to evaluate the protein functionality.

[0018] Also, the ~~effect for restoring~~ restoration of the cytotoxicity of NK cells *in vitro* is examined by using IL-6 and IL-15 together or ~~each alone~~ individually. *In vivo*, the expression of IL-6 and IL-15, the distribution of lymphocytes in the spleen and the cytotoxicity of NK cells are evaluated after introducing ~~of~~ a constructed plasmid of IL-6 and IL-15 into BALA/c mice. ~~Besides,~~ ~~t~~The C.B-17 SCID mouse is used as an animal model for testing the effect of cytokines on ~~the effect of~~ NK cell function and the subsequent activity of NK cells on preventing the growth of CTVT tumor cells, because the SCID mouse has an immunologic deficiency in B and T lymphocytes, ~~but keeping functional~~ while keeping NK cells functional. The effects of the present complex immuno-gene medical composition in activating NK cells and against CTVT are evaluated.

[0019] ~~From~~ ~~t~~The results of the above-described bioassays~~, it is demonstrated~~ demonstrate that the medical composition of the present invention is efficient in antagonizing the TGF-β inhibitory effect on NK cells, restoring an inhibited host immune system, activating NK cells in the host, and enhancing ~~ability~~ the effectiveness of the host immune system ~~in~~ against tumor cells. The medical composition including the combination of ~~the kinds of~~ Th1 and Th2 cytokines ~~is~~ acts against tumors not only by antagonizing TGF-β produced by the tumor cells ~~with~~ exhibiting low expression of MHC, but also by taking advantage of other immune responses. The present invention provides multiple strategies to inhibit growth of tumor cells.

[0020] Having ~~been~~ fully described the present invention, examples illustrating its practice are set forth below. These examples should not, however, be considered to

limit the scope of the invention~~,~~ as it is contemplated that modifications will readily occur to those skilled in the art, which modifications will be within the spirit of the invention and the scope of the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The related drawings in connection with the detailed description of the present invention ~~to be made later~~ are described briefly as follows, in which:

[0022] **FIG. 1** shows the influence of IL-6 on the cytotoxicity of NK cells. The E/T ratio is 13/1~~,~~. ~~t~~There are ~~triple~~ three samples in each treated group (N=3). The X-axis represents the concentration of IL-6; and the Y-axis represents the specific lysis percentage (cytotoxicity) of YAC-1 cells.

[0023] **FIG. 2** ~~shows comparison of~~ compares the effect of IL-15 and IL-2 ~~to restore~~ on restoring the ability of NK cellular cytotoxicity inhibited by TGF-β. The E/T ratio is 13/1~~,~~. ~~t~~There are ~~triple~~ three samples in each treated group (N=3). The X-axis represents the groups treated; and the Y-axis represents the specific lysis percentage (cytotoxicity) of YAC-1 cells. NC is the control group without treatment of cytokines.

[0024] **FIG. 3** shows the influence of the combined usage of IL-6 and IL-15 on NK cellular activity inhibited by TGF-β. The E/T ratio is 13/1~~,~~. ~~t~~There are ~~triple~~ three samples in each treated group (N=3). The X-axis represents the groups treated; and the Y-axis represents the specific lysis percentage (cytotoxicity) of YAC-1 cells. NC is the control group without treatment of cytokines.

[0025] **FIG. 4** shows the distribution of splenocytes after delivery of IL-6 and IL-15 plasmids into the BALB/c mice ~~body with~~ bodies via electroporation (N=3). A: Percentage of CD3+T cells in the treated groups. B: Percentage of CD19+B cells in the treated groups. C: Percentage of NK1.1+NK cells in the treated groups.

[0026] **FIG. 5** shows the NK cellular cytotoxicity of splenocytes after delivery of IL-6 and IL-15 plasmids into BALB/c mice ~~body with~~ bodies via electroporation (N=4). ▲ represents the group treated with a Mock vector; □ represents the group

treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with both IL-6 and IL-15 plasmids ~~together~~; ■ represents the group treated with both IL-6 and IL-15 plasmids ~~together and~~ as well as by the administration of anti-asialo GM1 antibody.

[0027] **FIG. 6** shows the influence of complex immuno-gene therapy on tumor establishment of CTVT. ▲ represents the group treated with a Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with both the IL-6 and the IL-15 plasmids ~~together~~; ■ represents the group treated with both the IL-6 and IL-15 plasmids ~~together~~ and with anti-asialo GM1 antibody ~~administration~~; x represents the group treated with both the IL-6 and IL-15 plasmid together ~~and~~ as well as with administration of normal rabbit serum.

[0028] **FIG. 7** shows the tumor growth curve of CTVT after therapy ~~of~~ involving complex immuno-gene adminstration. There are 6 mice in each treated group (N=6). ▲ represents the group treated with a Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with both the IL-6 and the IL-15 plasmids ~~together~~.

[0029] **FIG. 8** shows the influence of complex immuno-gene therapy on the survival rate of mice (N=5-6). ▲ represents the group treated with a Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with both the IL-6 and the IL-15 plasmids ~~together;~~ ■ represents the group treated with both the IL-6 and the IL-15 plasmids ~~together and~~ as well as with ~~administration of~~ anti-asialo GM1 antibody.

[0030] **FIG. 9** shows the influence of blocking ~~of~~ NK cellular function on the effect against CTVT tumor growth (N=6). + represents group treated with anti-asialo GM1 antibody; - represents the control group ~~being control experiment~~.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0031] Because it is difficult to extract the IL-6 gene from the body of canines, and because there is very little IL-15 mRNA in the tissues of a normal animal body, the sequence encoding human IL-6 (SEQ. ID. NO: 1) obtained from a IL-6 plasmid and a chimeric sequence encoding human IL-15 (IL-2 SP/IL-15 MP chimeric gene, SEQ. ID. NO: 4) are used in the present invention. The chimeric sequence contains an artificial sequence encoding the signal peptide of IL-2 (SEQ. ID. NO: 2) and a sequence encoding human IL-15 (SEQ. ID. NO: 3).

[0032] A ~~C~~commercial pcDNA3.1/V5-His-TOPO TA Expression Kit is ~~applied~~ used to clone and construct the plasmids containing IL-6 gene and IL-2 SP/IL-15 MP chimeric gene, respectively. The constructed plasmids are transformed into E. coli cells (for example, from One shot® TOP10 competent E. coli) according to conventional methods. Also, PCR restriction enzyme cleavage~~,~~ and DNA sequencing are employed to confirm the sequence. The plasmids with the confirmed sequence are amplified~~,~~ and purified with a Nucleobond AX plasmid purification kit (Macherey-Nagel, Durën, Germany).

[0033] Conventional MTS ~~test~~ testing is carried out to determine the activity of IL-6 in supernatant after transfection. The cell line of TF-1 (ATCC No. CRL-2003) which is dependent on IL-6 as a growth factor is employed to check the biological function of the IL-6 expressed by the constructed IL-6 plasmid. The supernatant obtained after tranfection of the constructed IL-6 plasmid into Balb/3T3 cells (ATCC No. CCL-163) stimulates proliferation of TF-1 cells. ~~And t~~There is no stimulating activity in the supernatant obtained from a transfection of the pcDNA3.1/V5-His-TOPO vector into Balb/3T3 cells (ATCC No. CCL-163) or Balb/3T3 cells cultured alone. The result demonstrates that the constructed IL-6 plasmid expresses IL-6 protein ~~with~~ having a biological function.

[0034] The method for evaluating the biological function of IL-15 expressed by constructed IL-15 is similar to the above method, but the cell line of TF-1 is replaced by HT-2 (ATCC No. CRL-2003) which is dependent on IL-15 as a factor for cell proliferation. The supernatant obtained from the culture of Balb/3T3 cells after

transfection of constructed IL-15 plasmid is added into the culture of HT-2 cells. The supernatant obtained after ~~tranfection~~ transfection of the constructed IL-6 plasmid into Balb/3T3 cells stimulates proliferation of HT-2 cells. ~~And~~ ~~t~~There is no stimulating activity in the supernatant obtained after transfection of the pcDNA3.1/V5-His-TOPO vector into Balb/3T3 cells or Balb/3T3 cells cultured alone. The result demonstrates that the constructed IL-15 plasmid expresses IL-15 protein ~~with~~ having a biological function.

[0035] Moreover, the effects of IL-6 and IL-15 on antagonizing the TGF-β inhibitory activity of NK cells are examined *in vitro*. ~~Comparing~~ Compared to the usage of either IL-6 or IL-15 alone, there is higher cytotocicity to YAC-1 mice lymphoma cells ~~in usage of~~ when IL-6 and IL-15 are used together. The result reveals that the usage of IL-6 alone recovers the function of NK cells inhibited by TGF-β, but IL-6 alone is not capable of activating NK cells and thus, the effect ~~of~~ on cytotoxicity is limited. In the same way, the usage of IL-15 alone ~~,~~ can activate NK cells, but it cannot remove TGF-β. Therefore, the activity of NK cells is inhibited and cannot be restored completely. ~~The usage of~~ Using IL-6 and IL-15 together provides IL-6 to antagonize TGF-β inhibiting the immunity of NK cells, ~~furthermore~~ as well as ~~,~~ IL-15 to activate NK cells. The cytotoxicity of NK cells is elevated effectively through the combined usage of IL-6 and IL-15.

[0036] For evidencing the effect of the combined usage of IL-6 and IL-15 plasmids, the constructed IL-6 and IL-15 plasmids are muscle ~~electropolated~~ electroporated alone or together into the body of BALB/c mice *in vivo*, and the splenocytes of the BALB/c mice are examined. The method of muscle electroporation ~~is~~ has recently been widely used in non-viral vector delivery in gene therapy ~~recently~~. ~~Comparing~~ Compared to viral vectors, the non-viral vectors are preferred because the advantages they provide ~~including~~ include high safety, low immune response, good efficiency in plasmid delivery, good protein expression, and ~~near~~ proximity to the body surface to ~~easily operate~~ enable easy operation. The results from ELISA show that expressions of IL-6 and IL-15 in host mice are successful.

[0037] Fourteen days after muscle electroporation, the numbers and cytotoxicity of NK cells in the treated mice spleens are elevated more obviously ~~in usage of~~ when the

IL-6 and IL-15 plasmids is used together than ~~the usage of~~ when either the IL-6 plasmid or the IL-15 plasmid are used alone. Moreover, the numbers and cytotoxicity of NK cells, ~~in usage of~~ when using IL-15 plasmid alone at E/T ratio of 50/1 and 12.5/1, are lower than ~~in usage of~~ when using the IL-6 and IL-15 plasmids together, but higher than in usage of IL-6 plasmid alone and in usage of vector. ~~It~~ This result reveals that IL-15 by itself is capable of enhancing ~~activating~~ the activation of NK cells.

[0038] —The C.B-17 SCID mouse is an artificial breeding strain ~~with~~having an immunodeficiency. The mouse has no mature T cells and no B cells with normal functionality, but it has normal myeloid cells, antigen-presenting cells (APCs) and NK cells. The mice are inoculated with CTVT for xenotransplantation. ~~And m~~Muscle electroporation is carried out to deliver IL-6 and IL-15 plasmids into the mice ~~body~~bodies. First, the influences of IL-6 and IL-15 on tumor establishment are evaluated. Secondly, the inhibiting effects of the two cytokines against established tumors are also evaluated. The experimental results demonstrate that combined usage of IL-6 and IL-15 plasmid is most effective in inhibiting the establishment of the tumor, and IL-15 plasmid alone is ~~little~~ not very effective ~~to inhibit~~ in inhibiting tumor establishment. No obvious influence ~~is observed in~~ due to the usage of IL-6 plasmid alone is observed. In addition, only the combined usage of IL-6 and IL-15 plasmid is effective to reduce the growth rate of the established CTVT. IL-6 plasmid or IL-15 plasmid alone cannot suppress the growth of ~~-~~an established tumor. In another experiment, anti-asialo GM-1 antibodies, an i.e., antibody that blocks the function of NK cells, is intraperitoneally injected into the SCID mice that carry the tumor. Then, the combined composition of IL-6 and IL-15 plasmid is ~~delivery~~ delivered into the mice. This combined composition cannot suppress the growth of CTVT. The result reveals that NK ~~cell plays~~ cells play an important role in such method of complex immuno-gene therapy.

Example 1: Preparation and synthesis of IL-6 and IL-15 genes

[0039] According to the mRNA sequence encoding human IL-6, Accession No. NM_000600 from Genbank, NCBI pubmed (http: // www. ncbi. nlm. nih. gov) , the IL-6 gene containing 636 bases (the sequence from the base 63 to 698 is ~~as~~ SEQ ID NO: 1) in its whole length is amplified and obtained with conventional methods.

[0040] The sequence encoding human IL-2 signal peptide (IL-2SP, 60 base, base 461 to 107, SEQ ID NO: 2) is linked to the sequence encoding human IL-15 mature peptide (IL-15MP, 342 base, base 461 to 802, SEQ ID NO: ~~2~~ 3) to form a IL-2 SP/IL-15 MP chimeric gene (SEQ ID NO: 4) according to the design presented by Kazuhiro et al. (2001). The design overcomes the disadvantage that it ~~is~~ operates via multiple regulators in transcription and translation and leads to low production of IL-15 protein. In amplification, the sense strand of chimeric gene sequence is synthesized with 13 primers, which primers are from the chimeric gene sequence, total length of 402 bases, fractionated ~~by~~ every 30 bases from the direction of 5'end. The antisense strand is synthesized with another 13 primers as the linked bridges, which sequences are complemented to the last and the next sense strain with 15 bases after the 15th base from the starting base.

[0041] The human IL-6 coding sequence is obtained from a PCR product amplified and purified from a IL-6 plasmid (from National Taiwan University College of Medicine, Taipei, Taiwan) which is created by inserting a human IL-6 gene into pcDNA3. The human IL-2SP/IL-15MP chimeric gene is obtained from a PCR product amplified with the aforementioned 26 primers and purified. The sequences of primers in the antisense strand are complemented to the last and next sense strain with 15 bases. The primer anneals to the sequence complementary to its own sequence in the reaction with DNA polymerase to form a template. The product of IL-2SP/IL-15MP chimeric gene is obtained from a PCR reaction.

Example 2: Cytotoxicity evaluation of NK cells in mouse spleen *in vitro*

[0042] The BALB/c mice, aged 6 to 8 weeks, are sacrificed and the spleens are taken. The spleens are homogenized in RPMI-1640 medium to obtain a single cell suspension. The cell suspension is centrifuged at 1,500 rpm and 4°C for 10 minutes. After discarding the supernatant, the cell pellets are added into 5 ml of 1 X ACK lysis buffer (10 X ACK lysis buffer contains 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA) to lyse red blood cells for 5 minutes at room temperature. The cell lysate is added into 10 ml of RPMI-1640 medium, centrifuged at 1,500 rpm and 4 °C for 10 minutes. After discarding the supernatant, the cells are rinsed once with RPMI-1640 medium ~~once~~. ~~Count~~ tThe cells ~~numbers~~ are counted and ~~dilute the cells~~ diluted in LAK medium which is RPMI-10 medium containing 50 μM 2-mercaptoethanol and

IL-2~~,~~ ~~t~~The ratio of IL-2 to cells is 500 U to 1 x 10^6 viable cells. The Cell solution is cultured with 2 x 10^6 cells/2 ml per well in culture 24 well-plate at 37 °C for 4 to 6 days in a 5% CO_2 incubator. ~~After~~ 3 days ~~from~~ after culturing, IL-2 is again added into the culture in a ratio of 500 U to 1 x 10^6 viable cells~~ again~~. To evaluate the influence of IL-6, IL-15, or TGF-β ~~onto~~ on the cytotoxicity of NK cells of mice splenocytes, the method for culturing of spleen cells is in accordance with the above description but the lymphokine is replaced by the cytokine ~~being interested~~of interest.

[0043] YAC-1 lymphoma cells (ATCC No. TIB-160) from mice are rinsed with PRMI-1640 medium twice with centrifugation at 1,500 rpm and 4°C for 10 minutes, and counted ~~with~~ using the Trypan Blue ~~Eexclusion~~ Exclusion Test. The cells are suspended in PRMI-1640 medium with 5 x 10^5 viable cells/ml, and the cell numbers are determined with effector cells. The fluorescence dye of 3,3'-dioctadecyloxacarbocyanine (DIOC 18, Sigma, MO, USA) is added into the cell suspension in a ratio of 10 μl to 5 x 10^5 viable cells/ml, and incubated at 37 °C for 16 hours in a 5% CO_2 incubator for reaction. After rinsing twice with centrifugation at 1,500 rpm and 4°C for 10 minutes, the cells are suspended into an RPMI-10 medium.

[0044] The splenocytes and YAC-1 cells~~, according to the mentioned treatments~~ treated as mentioned herein, are mixed ~~with~~ in various Effector/ Target ratios (E/T ratio), 50/1, 25/1, 12.5/1, 6.25/1, 3.125/1 and added into the round bottom wells ~~with round bottom~~ of a 96 well-plate (200 μl at most per well). The mixtures are centrifuged at 1,100 rpm and 4 °C for 5 minutes, and then incubated at 37 °C for 4 hours in a 5% CO_2 incubator in the dark. After incubation, the cells are harvested and mixed with propidium iodine (PI) (2500μm/ ml) ~~which~~ at a volume ~~is~~ of 1/100 of the cell solution. The cells are analyzed with flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, NJ, USA) and CellQuest software.

[0045] The results for IL-6 stimulating splenocytes of BALB/c mice show that the specific lysis percentage of YAC-1 lymphoma cells of mice ~~dose~~ does not increase with increased IL-6 dosage (**FIG. 1**). After allowing 6 days for activating splenocytes with cytokines, the specific lysis percentage in the group treated with 400U of IL-15 is similar to that of the group treated with 2000 U of IL-2. The cytotoxicity of NK cells is completely inhibited when the cells are cultured with both IL-2 and TGF-β,

but only partially inhibited when cultured with IL-15 and TGF-β together. The results reveal that IL-15 ~~itself~~ is superior to IL-2 for activating NK cells, and for restoring the NK cell cytotoxicity inhibited by TGF-β (**FIG.2**). Mice splenocyte culture with IL-6 and TGF-β together, with IL-15 and TGF-β or TGF-β alone, show that IL-6 is not capable of promoting cytotoxicity of NK cells, but IL-15 slowly promotes the NK cellular cytotoxicity inhibited by TGF-β. The most significant promoting effect is found when IL-6 and IL-15 are used together. (**FIG. 3**).

Example 3: Influence of IL-6 and IL-15 gene delivery with electroporation on cytotoxicity of NK cells from BALB/c mice

[0046] The BALB/c mice are divided into four groups for different treatments: (1) treatment with 100 μg of pcDNA3.1/V5/His/TOPO (Mock) vector; (2) treatment with 100 μg of IL-6 plasmid; (3) treatment with 100 μg of IL-15 plasmid; and (4) treatment with 100 μg of IL-6 plasmid and 100 μg of IL-15 plasmid together. There are 6 mice in each group. The solutions for treatment such as vector, IL-6 plasmid and IL-15 plasmid solution are prepared ~~as the~~ at a concentration of 1 mg/ml with saline before electroporation.

[0047] BALA/c mice are anesthetized and each mouse is injected with 50 μl of vector or plasmid solution into both sides of a muscle~~s~~, respectively. After ~~S~~standing for 2 minutes to diffuse the injected solution into the muscle~~s~~, the mice are electroporated with an electroporator (Electro Square Porator, BTX ECM 830)~~,~~. ~~and~~ tThe electroporation is carried out at 0.5 cm of inserting depth, at 100 volts for 10 times, and at 50 ms each time. After the electroporation treatments, the blood samples are taken on day 0, 3, 8, 12, 14, 15, 20, 25, 27 and 30. The blood samples are centrifuged to recover the serums and stored at -20 °C.

[0048] To examine the protein expression of the IL-6 and IL-15 plasmid~~s~~ *in vivo*, the commercial ELISA kit (IL-6: Endogen, MA, USA; IL-15: Biosource, CA, USA) is applied to serum samples to assay the concentration of IL-6 and IL-15 with a conventional procedure.

[0049] The results demonstrate both ~~two~~ plasmids express proteins in the mice bodies. IL-6 is detectable in serum samples in all groups, but the concentration and

~~expressed~~ expression times are not in accordance. IL-15 is detectable only in ~~the~~ groups (3) and (4).

[0050] On day 14 after electroporation, 4 mice are sacrificed for each group to collect the spleen cells and~~,~~ the lymphocytes subpopulations (T, B cells and NK cells) and the cytotoxcity of NK cells are evaluated.

[0051] Spleens taken from the electroporated mice~~,~~ are homogenized to obtain splenocytes. ~~A~~ 100 µl splenocytes suspensions of 1 x 10^7 viable cells/ ml are incubate~~d~~d with various monoclonal antibodies including rat-anti-mouse CD3-FITC antibody (Serotec, Oxford, UK), rat-anti-mouse CD19-FITC antibody (Serotec, Oxford, UK), rat-anti-mouse NK1.1-FITC antibody (PharMingen), and other isotypes. A 100 µl ~~of~~ splenocyte solution is added into 1 µg of antibody, reacted at 4 °C for 45 minutes, and then rinsed with 1 X PBS solution twice. To assess cell viability, 500 µg/ml of propidium iodine is added into the cell solution. The cell subpopulations are analyzed with flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, NJ, USA).

[0052] ~~From~~ Based upon the results of the above procedure, there are no obvious differences in percentage of T cells and B cells among the four experimental groups (**FIG. 4 A and B**), and the percentage of NK cells is significantly elevated in the treatment of group (4). In addition, group (4) shows superior NK cellular cytotoxicity ~~to~~ over the other groups in whatever E/T ratios. Group (3) exhibits significantly higher cytotoxicity ~~significantly~~ when the E/T ratio is 50/1 or 12.5/1, but there is no significant difference when the E/T ratio is 3.125/1. There is no significant difference between groups (1) and (2) in any E/T ratios (**FIG. 5**).

Example 4 : Effects of combined usage of IL-6 and IL-15 plasmids in inhibiting CTVT in C.B-17 SCID mice

[0053] CTVT is surgically excised from canines inoculated artificially, homogenized and passed through a two layer stainless mesh (No. 25) to obtain a single cell suspension. CTVT cells are isolated with 42% of Percoll (Amershampharmacia biotech, NJ, USA). Vital stain (Trypan B~~b~~lue Exclusion Test)

is applied to determine the viability of the tumor cells. C.B-17 SCID mice are subcutaneously inoculated with 1 x 10^8 viable CTVT cells with an 18 G syringe on each side of the back. The size of the tumors is measured twice a week after inoculation of CTVT. The tumor size is determined according to the following formula:

$V = \pi \times L \times W \times H / 4$

wherein, V is volume of tumor (cm^3), L is length of tumor (cm), W is width of tumor (cm) and H is height of tumor (cm).

[0054] Electroporation is performed on day 7 post CTVT inoculation (when the tumor is not established yet) to observe the influence of IL-6 and IL-15 on CTVT establishment. It shows that the tumors reach an observable size (diameter is about 2-3 mm) after 14 days from CTVT inoculation. In the group ~~of combined usage of~~ treated with both IL-6 and IL-15 plasmids, the tumors are obviously smaller than those found in other groups within the period of observation. The ~~G~~group treated with IL-15 plasmid alone shows a smaller tumor size than group treated with IL-6 plasmid alone and treated with the vector, but there is no significant difference among those treatments statistically ($p>0.05$). Tumor growth rate in the group treated with IL-6 plasmid alone is similar to the group treated with the vector, and there is also no significant difference between the two groups.

[0055] Anti-asialo GM-1 antibody is dissolved in 1 ml of solution suitable for injection. Each mouse is intraperitoneally injected with 30 μl of the anti-asialo GM-1 antibody twice a week to block the function of NK cells, and then electroporated with IL-6 plasmid and IL-15 plasmid. The result reveals the growth rate of tumor is dramatically increased, and the tumor size is bigger than the groups ~~with~~ subjected to other treatments.

[0056] ~~Besides~~Additionally, C.B-17 SCID mice inoculated with CTVT cells are electroporated when the tumor reaches 5 mm. IL-6 and IL-15 together effectively delay the growth rate of established tumors. Three tumors (sample size is 6, and each mouse is inoculated on two sides) disappeared and ~~do~~ did not grow again. In the period of observation, the average tumor size ~~of~~ with the combined treatment (IL-6 plus IL-15) is significantly smaller than in groups treated with vector, IL-6 plasmid or

IL-15 plasmid alone. IL-6 plasmid alone or IL-15 plasmid alone is not effective in inhibiting the growth of the tumors, and the sizes of tumors are similar to the group treated with vector (**FIG. 7**).

[0057] Furtheurmore, in order to realize the relationship between tumor inhibition effect shown by combined usage of IL-6 and IL-15 plasmid, and NK cells, 4 C.B-17 SCID mice are injected peritoneally with anti-asialo GM-1 antibody to block the function of NK cells. The results show that administration of IL-6 and IL-15 plasmid in the same level mentioned above is not able to suppress growth of tumors after injection of anti-asialo GM-1 antibody, and the survival rate is increased obviously (**FIG. 8** and **FIG. 9**). Thus, NK cells are the major cells to inhibit the growth of the tumor. IL-6 and IL-15 are effective cytokines to promote host NK activity in inhibiting the establishment of a tumor and ~~in~~ against an established tumor.

WHAT IS CLAIMED IS:

Canceled

~~1.A complex immuno-gene medical composition for inhibiting tumor cells, which medical composition at least comprises a therapeutic effective amount of plasmid comprising a DNA sequence (SEQ ID NO: 1) expressing IL-6 and a therapeutic effective amount of plasmid comprising a DNA sequence (SEQ ID NO: 2) expressing IL-15.~~

~~2.The medical composition of claim 1, wherein the tumor cell expresses low or no MHC.~~

~~3.The medical composition of claim 1, wherein the tumor cell produces TGF β.~~

~~4.The medical composition of claim 1, wherein the composition antagonizes PGF β inhibiting NK cells with IL-6 and activates NK cells to enhance immune system with IL-15 in host body to inhibit growth of tumor cells.~~

~~5.The medical composition of claim 2, wherein the tumor is primary breast carcinoma, advanced renal cell carcinoma, melanoma, proatate cancer, lung carcinoma, other tumor sources from colon, bladder, skin, endometrium, poultry T lymphoma, or CTVT.~~

~~6.The medical composition of claim 3, wherein the tumor is mammary tumor, thyreoglandular cancer, hepatocellular carcinoma or Meth-A tumor.~~

~~7.A method for inhibiting growth of tumor cells with complex immuno-gene composition, which method comprises administrating a therapeutic effective amount of plasmid comprising a DNA sequence (SEQ ID NO: 1) expressing IL-6 and a therapeutic effective amount of plasmid comprising a DNA sequence (SEQ ID NO: 2) expressing IL-15 to host to activate the NK cells and enhance cytotoxicity of the NK cells.~~

~~8.The method of claim 7, wherein the tumor cell expresses low or no MHC.~~

~~9.The method of claim 7, wherein the tumor cell produces TGF β.~~

~~10.The method of claim 9, wherein the tumor is primary breast carcinoma, advanced renal cell carcinoma, melanoma, proatate cancer, lung carcinoma, other tumor sources~~

~~from colon, bladder, skin, endometrium, poultry T-lymphoma, or CTVT.~~

~~11.The method of claim 10, wherein the tumor is mammary tumor, thyreoglandular cancer, hepatocellular carcinoma or Meth-A tumor.~~

~~12.The method of claim 7, wherein the plasmids are administrated by non-viral vector delivery or viral vector delivery.~~

~~13.The method of claim 12, wherein the plasmids are administrated by plasmid transformation, particle gum, or muscle electroporation *in vivo*.~~

~~14.The method of claim 12, wherein the viral vector is adenoviral vector, adenoviral associated vector, herpes viral vector, poxviral vector, or retroviral vector.~~

~~15.A complex immuno-gene medical composition for inhibiting tumor cells, which medical composition at least comprises a therapeutic effective amount of IL-6 and a therapeutic effective amount of IL-15.~~

~~16.A complex immuno-gene medical composition for inhibiting tumor cells, which medical composition at least comprises a therapeutic effective amount of vector comprising a DNA sequence (SEQ ID NO: 1) expressing IL-6 and a therapeutic effective amount of vector comprising a DNA sequence (SEQ ID NO: 2) expressing IL-15.~~

COMPLEX IMMUNO-GENE MEDICAL COMPOSITION FOR INHIBITING TUMOR CELLS

ABSTRACT OF THE DISCLOSURE

Disclosed is a complex immuno-gene medical composition for activating NK cells to enhance a host immune system. The composition ~~is usage of a combination of~~ includes a plurality of cytokines, ~~combined usage of the kinds of~~ including Th1 and Th2 cytokines. Th2 cytokines antagonize~~s~~ TGF-β inhibiting NK cells to disable the inhibition of the immune system~~,~~. ~~and~~ Th1 cytokines activate~~s~~ NK cells in a host to enhance the host's ability ~~fighting~~ to fight against tumor cells. By ~~means~~ use of the complex immuno-gene medical composition, removal of tumor cells is ~~expectable~~expected.